

**The Role of Cyclosporin A, Leptin, and FK-506 in *Leishmania*
major Infections in Mice**

For the Degree
Master of Arts

By
Shannon M. Potter

Under the Direction of
James K. Mitchell, PhD

Ball State University
Muncie, Indiana
May 2009

Table of Contents

Chapter 1: Abstract	Page 1
Chapter 2: Introduction	Page 2
Chapter 3: Literature Review	Page 5
<i>Leishmania</i>	Page 5
Figure 1	Page 7
The Immune Response	Page 9
Figure 2	Page 12
Immune Response to <i>Leishmania</i>	Page 14
Cyclosporin A	Page 17
FK506	Page 18
Figure 3	Page 20
Leptin	Page 22
Figure 4	Page 24
Chapter 4: Materials and Methods	Page 28
Mice	Page 28
<i>Leishmania</i> Infection and Maintenance	Page 28
Cyclosporin A	Page 29
Leptin	Page 30
FK506	Page 30
Control Mice	Page 31
Limiting Dilution Assay	Page 32
Flow Cytometry Analysis	Page 32
Chapter 5: Results	Page 35
Table 1	Page 36
Figure 5	Page 37
Figure 6	Page 39
Figure 7	Page 40
Figure 8	Page 41
Figure 9	Page 43
Figure 10	Page 44
Chapter 6: Discussion	Page 45
Chapter 7: References	Page 48

Chapter 1: Abstract

The immune response to some pathogenic microorganisms fails to protect the individual from severe infection and disease. Subsets of lymphocytes play a role in the outcome of an infection, particularly two subsets of T cells, called T-helper (T_H1 and T_H2) lymphocytes. When preferentially stimulated, the T_H2 cells are often inadequate or inappropriate in controlling certain microbes and as a result serious infection develops. The T_H1 response, on the other hand, may result in the resolution of the severe infection. In this study, we attempted to determine if leptin, cyclosporin A (CsA), and/or FK506 could switch the immune response in *Leishmania major* infected BALB/c mice from a T_H2 to a T_H1 type response thereby protecting them from severe disease. Groups of mice were infected with *L. major* and treated prophylactically with one of the above three drugs. The mice were compared to non-treated, infected animals using a variety of observations including ulcer development, footpad size, quantification of *L. major* in tissues and cytokine production. Data suggests that FK506 and CsA are very effective immunomodulatory drugs, while the amount of leptin is critical for the balance between T_H1 and T_H2 .

Chapter 2: Introduction

Leishmania spp. are parasitic protozoa that cause a spectrum of mild to severe cutaneous diseases, as well as systemic disease in which severe enlargement of the spleen, liver, and lymph nodes occurs. There are about 2 million new cases of leishmaniasis each year and a tenth of the world's population is at risk for this infection (1). This organism is found in Africa, the Middle East, South and Central America, and India. The protozoan is transmitted by the sand fly, a biting insect belonging to the *Phlebotamine* or *Lutzomyia* genera. Whereas *Leishmania major* causes cutaneous ulcers in humans, it causes a systemic disease in some strains of inbred mice and is similar to the severe disease caused by *L. donovani* in humans. Although leishmaniasis is an extremely prevalent and devastating disease among many people throughout the world, our ability to control this disease through drugs or pesticides has proven inadequate. Furthermore our understanding of the pathology of these diseases is limited, leaving many unanswered questions including mysteries about the immune response to leishmaniasis.

The immune response to some pathogenic microorganisms, like *Leishmania spp.*, sometimes fails to protect the individual or an entire strain of animals from severe infection and disease. Although an immune response is mounted, it is not protective or curative, it becomes destructive, causing a systemic infection. When preferentially

stimulated, the T_H2 cells are often inadequate or inappropriate and result in a failure to control certain microbial infections. The T_H1 response, on the other hand, may result in the resolution of the infection. The balance between these two subsets often determines if an infection progresses or resolves itself. This balance is controlled by cytokines secreted by the two different subsets of lymphocytes and other cells in the body. Cytokines interact with cellular surface receptors and direct the cells in what to secrete, produce, or stop producing. Essentially, the T_H1 subset is stimulated by cytokines produced and secreted by other T_H1 cells and inhibited by cytokines produced and secreted by T_H2 cells. The opposite is true for the stimulation and inhibition of the T_H2 subset.

An inbred strain of mice, called BALB/c, is very susceptible to *Leishmania major*. It is believed that the resulting severe illness is due to the type of response these mice mount against the pathogen. It has been shown that these mice mount a T_H2 type response resulting in the production of antibodies and B cells that are ineffective against the intracellular parasite. It is believed that if this response can be suppressed or reversed, and if a T_H1 type response is stimulated, increasing cell-mediated immunity, these animals would not succumb to the illness. We attempted to accomplish this by treating the mice prophylactically with cyclosporin A (CsA), leptin, or FK506. CsA and FK506 are commonly regarded as immunosuppressants and leptin, a naturally occurring hormone, is an appetite suppressant with immunomodulatory properties. Preliminary work in our laboratory has suggested that these treatments alter the susceptibility of these

mice and makes them more resistant to severe disease following infection with *L. major* (2).

Chapter 3: Literature Review

Leishmania

Leishmania species are obligate intracellular parasites that are found throughout the world. Different *Leishmania* species cause a variety of infections in humans including the frequently fatal visceral leishmaniasis or Kala Azar, self healing tropical sores or disfiguring mucocutaneous leishmaniasis. Visceral leishmaniasis in humans is characterized by fever, weight loss, hepatosplenomegaly, and anemia and is caused by *L. donovoni*. It is often fatal even following treatment and is persistently fatal without treatment (1). Mild cutaneous leishmaniasis is characterized by a skin ulcer at the site of the sandfly bite. It is usually self-healing within three to six months. A rare form of this disease, called diffuse cutaneous leishmaniasis, presents as disseminated skin lesions. An uncommon consequence of cutaneous leishmaniasis, which can present itself years after the initial skin ulcer heals. It is a metastatic complication of the primary lesion resulting in disfiguring and progressive ulceration at the nasal mucocutaneous junction (1). This is called mucosal leishmaniasis.

These diseases are extremely prevalent in Africa, the Middle East, parts of Europe, Asia, and in Central and South America. They are transmitted by the sand fly, a biting insect belonging to the *Phlebotomine* or *Lutzomyia* genera. Although *Leishmania*

major causes a cutaneous form of leishmaniasis in humans, in the mouse it serves as an animal model for the severe systemic form of the disease.

The sandfly's natural hosts are rodents, small mammals, and dogs; humans are accidental hosts (1). Upon biting an infected host, the sandfly ingests the amastigote form of the parasite. The amastigotes migrate to the hindgut and transform into promastigotes, which undergo asexual reproduction (3). The promastigotes are the infective form of the parasite. The promastigotes then migrate forward to the esophagus of the sandfly, clogging the esophagus. They are inoculated into the victim's blood stream when the sandfly bites and forces the blockage from its system. The promastigotes are ingested by the victim's macrophages and undergo another transformation back into amastigotes. The amastigotes asexually reproduce within the macrophage and cause the infected macrophage to rupture. The released amastigotes are engulfed by other macrophages and the cycle repeats itself (3).

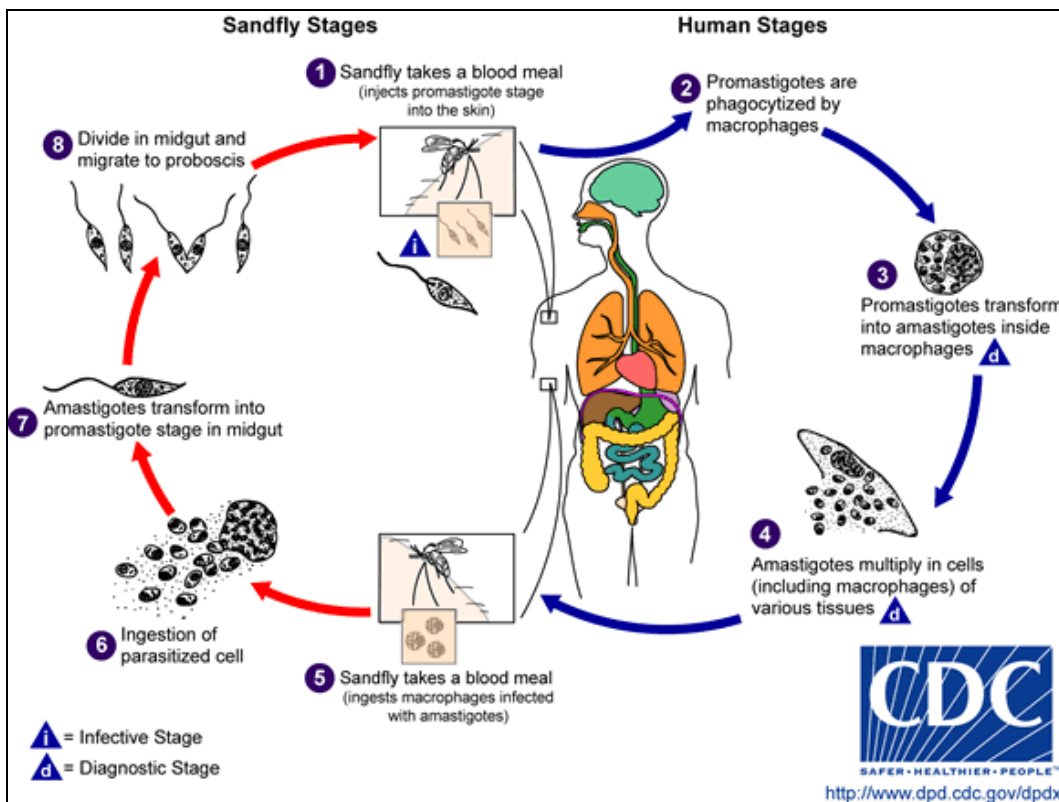


Figure 1: The life cycle of *Leshmania* spp. The sandfly injects the promastigote form of the parasite into the skin. Macrophages ingest the promastigotes. The promastigotes transform into amastigotes and multiply in the body's cells. The sandfly ingests macrophages infected with amastigotes, the amastigotes transform into the promastigote stage in the sandfly's midgut where they multiply. The promastigotes then migrate to the proboscis and the cycle repeats (47).

L. major uses the host complement receptors to gain access to the hostile environment of the phagolysosome. Activated macrophages and T-lymphocytes are recruited to the site of infection (4). Once inside the phagolysosome, *L. major* utilizes the host-cell's cyclophilin proteins as part of their replication mechanism (5). Apparently, in humans and animals, the outcome of the disease is correlated with the type of T-helper response. Resistance is associated with the T_H1 response; susceptibility is associated with the T_H2 response. The T_H1 lymphocytes primarily produce $INF-\gamma$ and IL-2 and the T_H2

lymphocytes primarily produce IL-4 and IL-10. The T_H1 response induces a cell mediated (CMI) response whereas the T_H2 response induces humoral immunity. CMI is the more effective response toward *Leishmania* because it is an obligate intracellular parasite in animals. It appears that a humoral response has little or no effect on the parasite itself because the antibodies produced cannot bind to the intracellular parasite. Indeed, even in the presence of high antibody levels, severe systemic disease can develop. The depressed T_H1 response and the activated T_H2 response is an important factor in the visceral leishmaniasis disease process. This apparently leads to hyperglobulinemia, the presence of excessive amounts of antibodies in the blood stream, a hallmark characteristic and diagnostic of visceral leishmaniasis (3).

Visceral leishmaniasis has become increasingly prevalent with the spread of HIV (1). With the onset of AIDS, previously “healed” or asymptomatic systemic *Leishmania* infections reactivate. Species that would normally cause only the cutaneous form of the disease may present with the visceral form. Co-infections of *Leishmania* and HIV are often resistant to treatment and substantially accelerate the progression of AIDS (1).

Diagnosis of cutaneous leishmaniasis is accomplished by taking scrapings from the edge of an ulcer, staining them with Wright’s or Giemsa’s stain and observing the smear microscopically for the parasite in the endothelial cells and the monocytes (3). The parasites usually cannot be found in circulating blood. Cultures are also made in

case the amastigotes go undetected in the smear from the scrapings (3). Treatment is usually unnecessary because this form of the disease is normally self-healing.

Diagnosis of visceral leishmaniasis is accomplished by several techniques. Microscopic examination of spleen, liver and other visceral tissues for amastigotes is a primary technique (3). Although the production of antibodies by infected individuals does not help cure the disease, it is useful in the diagnosis of the disease. Individuals have a high titer of IgM, IgG as well as IgE antibodies. Because of the presence of these antibodies, ELISA and indirect fluorescent antibody techniques may be used as a diagnostic technique. Treatment of visceral leishmaniasis is often sodium stibogluconate or pentamidine isethionate (3). Amphotericin B/lipid formulation has been shown to be an effective treatment, however it is expensive. Miltefosine, a more affordable treatment, and is being used more commonly, however it is not as effective as the more expensive treatments (3). These treatments are not only expensive, but they must be administered for several weeks and often have serious toxic side effects (3).

The Immune Response

Immunity to pathogenic microorganisms is characterized by both innate resistance and acquired immune responses. (6). Innate immunity is not inducible, is always present, and is an important but general defense system against pathogens. It includes physical barriers like skin, hair, and mucosa, and chemical barriers like lysozymes, and stomach acids. Innate immunity is not directed against a specific foreign agent (e.g. pathogen). Acquired immunity, however, is gained through immune responses to a specific foreign

substance or antigen. It is induced and stimulated by these antigens or immunogens, it can change over time and is the basis for vaccinations. Acquired immune responses are induced and directed against a specific foreign agent. Essentially acquired immunity has the ability to distinguish self from non-self, and is characterized by its inducibility, specificity, memory, and regulation (6).

Acquired immunity is further broken down into two branches, humoral immunity and cell mediated immunity (CMI). Humoral immunity is primarily mediated by antibodies present in the plasma, lymph and tissue fluids (7). It is particularly protective against extracellular pathogens and toxic macromolecules. There are primarily three types of cells involved in the immune response: antigen presenting cells, T helper lymphocytes and B cells which actually secrete the antibody. B cells synthesize and display membrane-bound immunoglobulin, or antibody (Ab) molecules which serve as receptors for antigens. Following stimulation with antigen and T cell lymphokines, B cells differentiate into plasma cells which secrete antibodies, but do not express them on their membranes. Together these cells, and molecules make up the humoral immune response. B cells as well as dendritic cells and macrophages also serve as antigen presenting cells (APC), which phagocytize antigens, break them down and present the antigen to T cells on major histocompatibility complex molecules (MHC). T cells can only recognize antigen after it is processed and displayed on an MHC class I or II molecule.

MHC molecules interact with T cells, via the T cell receptor (TCR). The TCR is the T cell's membrane bound antigen receptor. Unlike the B cell's membrane bound receptor, the TCR cannot recognize free antigen. Instead it recognizes antigen bound to MHC molecules. There are two major types of mature T cells, characterized by expression of either $CD4^+$ and $CD8^+$ molecules on their cell surfaces (7). $CD4$ and $CD8$ are cell surface markers/receptors.

Functionally these cells behave differently. $CD8^+$ T cells bind with antigens presented on MHC class I molecules (7). These cells generally function as cytotoxic T cells (T_C cells) killing virally infected cells. These cells generally recognize endogenous peptide antigens. $CD4^+$ T cells bind with antigens presented on MHC class II molecules. These cells generally function as T-helper cells (T_H cells) helping either other T cells or B cells become effector cells. There are two types of T_H cells, T_{H1} and T_{H2} cells differentiated by the lymphokines they secrete. T_{H1} clones are involved in cell mediated immunity and T_{H2} clones are predominantly involved in humoral immunity, providing cognate B cell help (7). The two T-helper subsets are controlled by many different cytokines. The regulation of these subsets is shown in Figure 2 (7). The subsets themselves produce very specific cytokines which, in turn, regulate a specific set of immune cells directing the immune response toward humoral or cell-mediated.

The balance between T_{H1} and T_{H2} often determines the outcome of an infection. In many chronic illnesses, a polarized T_{H1} or T_{H2} type response occurs, which is

The diagram illustrates the differentiation and regulation of Th1 and Th2 cells. At the top, a yin-yang symbol represents the balance between Th1 (purple) and Th2 (red) pathways. Macrophages and NK cells, T_H2 cells, and mast cells are shown influencing this balance. Regulatory T_H cells produce TGF-β, which inhibits both Th1 and Th2 cells. Th1 cells are induced by IL-12 and IFN-γ, while Th2 cells are induced by IL-4. Both Th1 and Th2 cells produce IFN-γ, which inhibits the other. Th1 cells produce IL-2, IFN-γ, and TNF-β, which activate B cells, T_C cells, and macrophages. Th2 cells produce IL-4, IL-5, IL-6, IL-10, and IL-13, which activate B cells, mast cells, and eosinophils. The final outcomes are IgG2a production from B cells, T_C activation, macrophage activation, IgM/IgG1/IgA/IgE production from B cells, mast-cell growth, and eosinophil growth and differentiation.

Respiratory syncytial virus (RSV) causes most cases of viral bronchiolitis, which is the single most common cause of hospitalization of infants and young children (10). In

the 1960s children were vaccinated with a formalin inactivated virus preparation. Unfortunately, this vaccination enhanced the disease process when the child was exposed to the virulent virus. The killed virus induced a humoral type response instead of inducing a CMI type response. Therefore, the memory acquired by the immune system in these vaccinations was a humoral type immunity, which is directed by the T_H2 subset. This apparently led to an exacerbated inflammatory response when an RSV infection occurred. Resolution of the infection is associated with $CD4^+$ and $CD8^+$ T cells that produce $INF-\gamma$. In these resolving infections, only low levels of IL-2, IL-4 and IL-5 were detected which suggests a T_H1 type immune response. Interestingly, *in vitro* studies show the co-production of IL-4 and $INF-\gamma$, suggesting that the immune response is not always completely polarized (10). Field et. al claim that the only evidence for the presence of a pure T_H1 or T_H2 type response is based on clonal proliferation of cells obtained from the peripheral blood of patients (11). In most situations, a mixture of responses exists with shifts in either direction.

The outcome of an HIV infection also seems to be dependent on the type of T_H response. The presence of specific T_H1 type responses in individuals has been shown in persons who have been exposed to HIV but are not seropositive for HIV (12). This suggests the presence of HIV-specific protective immunity in these individuals. Persons who live in areas where infectious diseases are endemic seem to have a highly activated immune system. Their peripheral blood mononuclear cells were found to secrete significantly higher amounts of IL-2, IL-4, IL-10, and soluble TNF receptors and lower

amounts of IL-6 in comparison with healthy non-Ethiopian controls (12). The people of these countries (Africa, the middle East, parts of Europe, Asia, and Central and South America) are often exposed to helminthic infections early in life (3). It is believed that the helminth infections alter the balance between T_H1 and T_H2 in such a way that makes the host more receptive to HIV and more vulnerable to its effects (12). The same is true for mycobacteria infections and other intracellular parasitic infections, like leishmaniasis (13). However, Maizels et. al. (14) show that helminthic infections steer the immune response of the host in the direction of T_H2 , which is more protective for the host and down regulates the T_H1 response. Since helminthes are endemic to areas of the world that are also endemic to *Leishmania spp.*, Maizels et. al.'s (14) determination could explain why some people are immune to leishmaniasis and others become seriously ill.

Immune Response to *Leishmania*

Murine models have been very useful in understanding the visceral disease process. Mice resistant to *L. major* develop a protective immunity by the expansion of $CD4^+$ T_H1 T cells. These protective T_H1 cells result from their production of INF- γ , which induces the synthesis of nitric-oxide synthase, (iNOS) (4). Nitric oxide synthase leads to the L-arginine-dependent production of reactive nitrogen radicals, which are toxic to the intracellular *Leishmania* parasite. *In vitro*, this cytotoxicity is dependent on a functional Fas-FasL pathway (4). Stenger et. al. have shown that the cutaneous lesion and draining lymph nodes of infected, clinically resistant C57BL/6 mice have a detectable amount of iNOS earlier during infection and in significantly higher amounts than in

infected, susceptible BALB/c mice (15). Cells isolated from lesions in infected BALB/c mice were found to stain positively for transforming growth factor beta (TGF- β), a powerful inhibitor of iNOS *in vitro* (15). Susceptible mice develop an early expansion of T_H2 T-cells. These cells produce IL-4, which appears to be the major inducer for T_H2 and inhibitor for T_H1 type responses (16). Treatment of BALB/c mice with anti-CD4 induced resistant to the disease, and restored the expression of iNOS in the tissue (15). This response is thought to be due to a parasite antigen that is a *Leishmania* homolog of receptors for activated C kinase, or the LACK antigen. Mice that have been transgenically made to express the LACK antigen in the thymus exhibit a diminished T_H2 response and a healing phenotype (16). The type of response that dominates the outcome of the infection occurs very early after detection of antigen, like *Leishmania*'s LACK antigen. CD4⁺ cells specific for the *L. major* LACK antigen were shown to expand 100-fold; 70% of those cells were detected to as producers of IL-4 by hour 96 (17). BALB/c mice have been specifically shown to produce a burst of IL-4 within the first day of *L. major* infection (18). This early IL-4 production was shown to be a result of the cognate recognition of a single epitope in a distinctive antigen, the LACK antigen (18). Schilling et. al. (19) have demonstrated that LACK-specific T-cells are active in both the draining lymph node and the site of infection, further promoting the rapid replication of the parasite and its dissemination to the internal organs.

The induction of T_H1 dominated or T_H2 dominated responses involves a very complex interaction between antigen presenting cells, specific antigen epitopes (such as the LACK antigen) and T cells. In addition to antigen TCR processing recognition, these interactions involve a number of cell surface costimulatory molecules such as CD40, CD28, B7 molecules and ICOS. Inducer costimulator proteins (ICOS) found on the surface of T cells have an important role in T cell induction, stimulation differentiation, as well as Ig class switching (20). ICOS is induced by a homolog of CD28 and reacts with B7 homolog. It enhances T cell cytokine production, up-regulates the production of the CD40 ligand and induces Ig production by B cells. ICOS is induced upon T cell receptor engagement with the antigen (20). Whereas another T cell co-stimulator molecule, CD28 is constitutively expressed on most T cells (7). Expression of ICOS is enhanced by CD28 costimulation. The inducible expression of ICOS suggests that ICOS regulates recently activated effector T cells. Functional studies have suggested that ICOS is important for the T_H2 response, preferentially inducing IL-4 and IL-10 production. In contrast, other studies suggest that ICOS may also regulate the T_H1 responses. Greenwald et. al. (20) demonstrated that ICOS^{-/-} mice infected with *Leishmania mexicana* exhibited pronounced deficits in Ig class switching and T cell cytokine production. Development of cutaneous lesions and inflammatory infiltrates was impaired in these mice. This demonstrated that ICOS is probably a critical regulator of both the T_H1 and T_H2 type responses in leishmaniasis. It is clear that much more needs to be understood about the pathology and immune response to leishmanial infections.

Cyclosporin A

Cyclosporin A (CsA) is a cyclic polypeptide metabolite commercially synthesized by the fungus *Tolypocladium inflatum* with a molecular weight of about 1200 which has been shown to have immunosuppressive effects (21). CsA is a lipophilic compound that contains neither free amino nor carboxylic groups. It has a formula of $C_{62}H_{111}N_{11}O_{12}$ and is a cyclic undecapeptide composed of ten amino acids and the unique amino acid N-methyl-2-amino-3-hydroxy-4-methyl-octa-6 enoic acid (MeBmt). With the exception of one D-alanine, all of the amino acids are L-series amino acids. Seven of the amino acids have an N-methylation, which is responsible for its lipophilic nature. Stabilization of the CsA molecule is maintained by several hydrogen bonds. CsA is synthesized in a non-ribosomal biosynthesis pathway (21). A non-ribosomal pathway is directed by a multienzyme template and without the direct use of nucleic acids (22). Biosynthesis using a non-ribosomal pathway is accomplished by the formation of linear peptidyl intermediates which may be enzymatically modified (22). It is thought that CsA is biosynthesized in a three step process: i. Synthesis of all building units, ii. Activation of the 11 amino acids, and iii. N-methylation and peptide formation (21).

Toxicity studies show that the selectivity of CsA is for lymphocytes and that there is a lack of effect on haematopoiesis (21). CsA acts selectively on lymphocytes, mainly T cells, affecting particularly the inductive phase of immunity, rather than the proliferative phase of immunity. CsA has been shown to alter the balance between T helper

lymphocytes and suppressor T-cells. Its immunomodulatory effects appear to result from the inhibition of the production of T-cell derived soluble mediators like IL-2 and IL-3 and INF- γ which leads to an inhibition of primary T_H cell activation and blocks the formation of lymphokines like IL-2. It also decreases the secretion of INF- γ . The production of IL- α and INF- β , however does not seem to be affected (21). T-cell proliferation is inhibited by the blockage of Ca²⁺ dependent pathways required for the induction of IL-2 transcription (23). Complexes of CsA inhibit the calmodulin dependent protein phosphatase, calcineurin (24). The calcineurin pathway dephosphorylates the inactive cytosolic form of the T cell specific nuclear factor NF-AT, which is a transcription factor necessary for T cell activation, See Figure 3 (7).

CsA is most commonly used as an immunosuppressant for transplant patients. Previous work in our lab has shown that in addition BALB/c mice treated prophylactically with CsA have significantly enhanced resistance to *L. major* infections (2).

FK506

FK506 is a macrolide that has a molecular weight of about 850 Daltons and is produced by a strain of the bacterium *Streptomyces* (25). It has a molecular formula of C₄₄H₄₉NO₁₂H₂O. FK506 has been shown to suppress the immune response both *in vivo* in mice and humans and *in vitro* it is more potent/mg than that of CsA, and has been

shown to suppress the immune system *in vitro* at about 100 times lower concentration than CsA. It also appears to be less toxic than CsA (26).

FK506, like CsA, has been shown to strongly suppress the humoral immune response, possibly by inhibiting the production of T-cell derived soluble mediators (26). FK506 especially affects mature CD4⁺ T cell reconstitution in the periphery, seemingly by interfering with thymic activation signals such as CD69, which is required for final maturation of T cells (27). Both compounds have also been shown to semi-selectively spare CD8⁺ T cells (25). These T cells are thought to produce cytokines that act on other lymphocytes; for example, these cells produce INF- γ , which stimulates the T_H1 response (26).

FK506 binds to an immunophilin named FKBP-12, or FK506 binding protein number 12 (28). Immunophilins serve as receptors to immunosuppressive or immunomodulatory drugs. FK506 has also been shown to target the calcineurin pathway in a similar manner as Cyclosporin A (Figure 3) (24, 29). The FK506/FKBP-12 complex targets the calcium dependent protein calcineurin. This complex inhibits phosphorylation of calcineurin. This inhibition increases the phosphorylation of NOS, a substrate for calcineurin; calcineurin dephosphorylates the NOS protein. The phosphorylated calcineurin enzyme diminishes its catalytic activity, suppressing the formation of NO₂, which mediates glutamate neurotoxicity. Tao and Aldskogius (28) have shown that FK506 enhances the survival of damaged nerves by the same pathway. Nitric oxide

(NO) inhibition prevents the glutamate-elicited neurotoxicity. FK506 may exert survival-promoting effects by decreasing NO synthesis via the inhibition of NOS, which is dependent of calcium for its activation (28). Down-regulation of NO, in turn, inhibits glutamate release and protects against neurotoxicity.

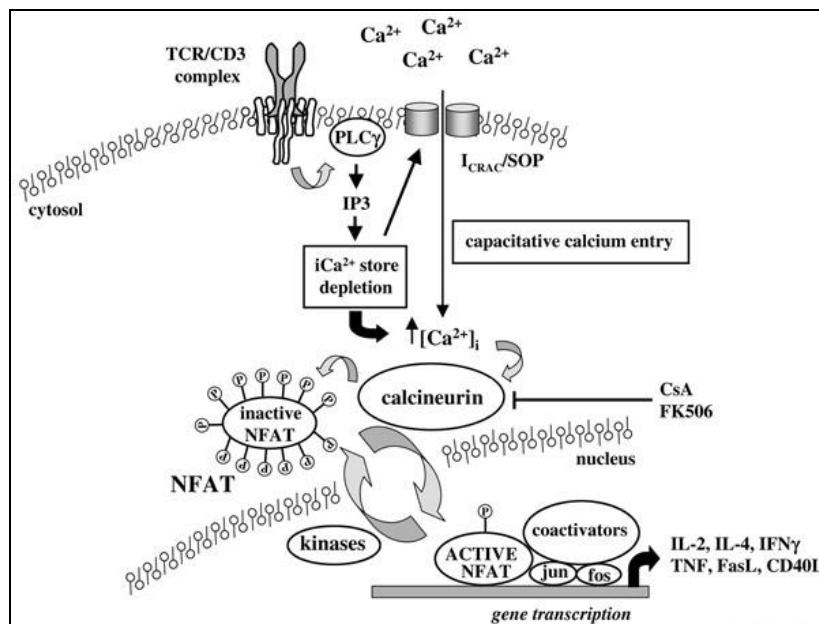


Figure 3: The Calcineurin Pathway and the Role of CsA and FK506: Calcineurin dephosphorylates inactive NFAT, which activates the transcription of cytokine genes. CsA and FK506 have been shown to block this pathway by inhibiting the dephosphorylation of Inactive NFAT. This, in turn, inhibits the transcription of cytokine genes (5).

Calcineurin also dephosphorylates the cytoplasmic nuclear factor of activated T cells, called NF-ATc (30). This induces its nuclear translocation and eventually forms a functional transcription factor that activates the transcription of IL-2. Therefore, FK506 also inhibits the synthesis and subsequent secretion of IL-2, which is the cytokine that induces the clonal proliferation of T cells by suppressing calcineurin's phosphatase

activity. Calcineurin has been shown to co-localize with FKBP-12 in neurons (31). This co-localization in the nerve, along with the inhibition of calcineurin by FK506 is believed to result in the promotion of neuroregeneration by enhancement of growth-associated protein-43 (GAP-43) (32). GAP-43 is a phosphoprotein that has a key role in nerve growth, which facilitates neuronal growth after injury.

Agostinho and Oliveira (33) studied the effect that FK506 and CsA has on diseases that are caused by prions in the brain. Prions are proteins that are enriched with β -sheet conformations, which makes them resistant to proteolytic cleavage by proteases. Alzheimer's disease, the most common form of dementia in the elderly, has been shown to be associated with the amyloid beta protein ($A\beta$) (34). The $A\beta$ protein results from the abnormal proteolytic cleavage of amyloid precursor protein. Agostinho and Oliveira (33) also studied prion-related encephalopathies (PRE), which are caused by the scrapie isoform of prion protein (PrP^{Sc}). Plaques formed by the presence of prions consist of a core surrounded by degenerated neuritis, microglia and astrocytic processes. The apoptotic pathway of neuronal death appears to be a prominent feature of both Alzheimer's disease and PRE. Apoptosis is thought to be regulated by the calcineurin pathway through certain cysteine aspartate-specific proteases (caspases). Caspase-3 is thought to be a major player in this apoptotic pathway. Agostinho and Oliveira (33) used FK506 to determine if the calcineurin pathway is involved in neuronal death induced by the $A\beta$ prion and the PrP^{Sc} prion. They found that FK506 prevented the release of cyt *c*

and thereby prevented the activation of caspase-3. This indicated that this phosphatase is involved in the neurotoxic mechanism induced by A β and PrP^{Sc}. These studies also indicated that FK506 did prevent neuronal cell death.

FK506 is currently being used as an immunosuppressant in transplant patients. It has been shown to reduce graft vs. host disease or rejection, which CsA does not (27). Previous work in this laboratory has shown that FK506 also significantly enhances the resistance to *Leishmania major* infections in BALB/c mice (unpublished data).

Leptin

Leptin is a recently discovered hormone that is secreted exclusively by adipocytes. It is a 16-kDa nonglycosylate peptide hormone (35). The primary amino acid sequence of leptin indicates that it may adopt a helical cytokine structure similar to IL-2 (36). The leptin receptor is expressed not only within the nervous system, but also within the immune, hematopoietic, and reproductive systems (37). Leptin induces proliferation, differentiation, and functional activation of hemopoietic cells (38). The leptin receptor has a sequence homology to the members of the cytokine receptor superfamily (39). It also increases the expression of the early activation marker CD69 in monocytes but not in lymphocytes. Cytokine production is enhanced, and the basal expression of activation markers such as CD38 in human monocytes are stimulated as previously described in the LPS stimulation system (33). Leptin, when given to lean or obese mice, increases basal metabolic rate and reduces food intake, leading to weight loss.

Leptin has been shown to be essential in balancing the susceptibility to infection and autoimmunity, as shown in Figure 4 (39). Normal leptin levels balance the T_H1 and T_H2 response, whereas if leptin levels fall too low, T_H1 type responses are reduced and T_H2 type responses are increased. If too much leptin is present there is a reduced T_H1 type response and an altered inflammatory response. Circulating levels of leptin are proportional to fat mass, but may be lowered rapidly by fasting or increased by an inflammatory response (39). An impaired cell mediated type response, or a T_H1 type response, and reduced leptin levels are both features of low body weight. Starvation or malnutrition, which decreases leptin levels, results in the suppression of T_H1 mediated inflammatory responses (40). It is well established that nutritional deficiency impairs cell mediated immunity, phagocyte function, and cytokine and antibody production (41).

Both naturally leptin-deficient mice and leptin-receptor-deficient mice, despite their great body-fat mass, have thymic and lymph node atrophy, reduced numbers of

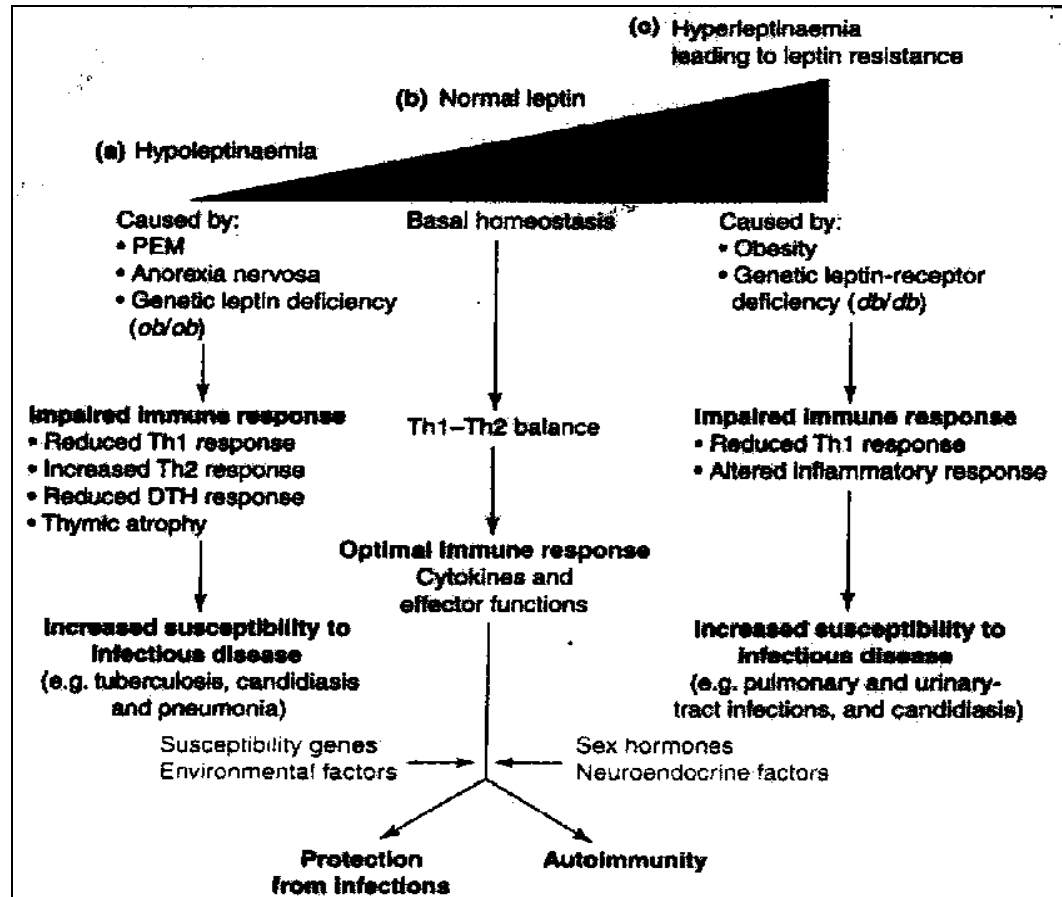


Figure 4: Leptin's effect on T_H1 vs. T_H2 balance. Normal levels of plasma leptin promote a balance between T_H1 and T_H2 type responses. Too much leptin or too little leptin cause this balance to shift (39).

bone-marrow precursors, and impaired cell mediated immunity (39). In rodents, it has been shown that after immunization females have a more vigorous T cell and antibody response than males. The presence of estrogens produce higher levels of T_H1 cytokines and they show a consistent *in vitro* secretion of $INF-\gamma$ and IL-1 (42). Androgens and testosterone produced by males increase the production of IL-4 and IL-5 and switch to the T_H2 type response (42). Serum levels of leptin are sexually dimorphic, being higher in

females than in males (42). This suggests that leptin is involved in gender-related differences in susceptibility.

Human leptin has been shown to enhance cytokine production by murine peritoneal macrophages. Santos-Alvaraz et. al. (37) have demonstrated that human leptin stimulates proliferation in a dose-dependent manner and functionally activates human circulating monocytes *in vitro* by inducing the production of cytokines such as TNF- α and IL-6. The expression of different activation markers in human monocytes, like CD25 (IL-2 receptor), CD71 (transferring receptor), and CD38 were also increased by leptin in a dose-dependent manner. The expression of other activation markers that were already present at high levels on the surface of resting monocytes, (e.g. HLA-DR, CD11b, and CD11c), were also enhanced by leptin in a dose-dependent manner (37). Santos-Alvaraz et. al. (37) also demonstrated that the stimulation of PBMC with leptin produced an increase in the percentage of monocytes producing cytokines. The effect of leptin was more evident on the population of monocytes producing IL-6, from which about 50% co-expressed TNF- α . However, the population of monocytes producing only TNF- α was less affected. Therefore a greater effect of leptin on monocyte IL-6 synthesis might be expected. The pro-inflammatory response of these cytokines may be enhanced by leptin, functioning as an amplification signal for the activation of monocytes. Monocytes are activated by leptin through the induction of proliferation expression of activation markers, and the production of cytokines.

Martín-Romero et. al. (38) have shown that leptin modulates CD4⁺ T lymphocyte activation toward the T_H1 phenotype by stimulating the synthesis of IL-2 and INF- γ . Leptin receptors are expressed on both CD4⁺ and CD8⁺ cells, as well as hemopoietic cells, other lymphocytes, and adipocytes. The leptin receptor has a sequence homology to members of the cytokine receptor class I superfamily (44). The receptor has been shown to have similar signaling capabilities to that of the IL-6 type cytokine receptor (45).

Martin-Romero et. al. (38) attempted to determine the effects of human leptin on activation, proliferation, and cytokine synthesis in peripheral human lymphocytes. They found that a leptin dose-dependent enhances the proliferation and activation of circulating T-cells when they were costimulated with either PHA or ConA. Alone, leptin was unable to activate T-cells. Leptin also dose-dependently enhanced stimulated CD69 expression; late activation markers CD25 and CD71 were also activated by leptin stimulation. Leptin modulated CD4⁺ T-cell activation toward the T_H1 phenotype by stimulating the production of IL-2 and INF- γ . This shift toward the T_H1 phenotype may have some relevance in the pathophysiology of immunologic alterations related to obesity. Upon measuring the levels of IL-2, INF- γ , and IL-4, they found that IL-2 and INF- γ levels were increased and that IL-4 was undetectable under the experimental conditions investigated. This suggests that leptin levels in the blood stream can easily shift the T_H1/T_H2 balance.

According to Lord et. al. (46), "Leptin increases the T_H1 and suppresses the T_H2 cytokine production. Without leptin present, the T_H2 response is favored; leptin seems to

induce the switch to the T_H1 response". Leptin modulates the production of IL-2 and INF- γ , which are T_H1 activators (38). Circulating levels of leptin have been found to be proportional to fat mass (43). These levels may be lowered by rapidly fasting or increased by inflammatory mediators (47, 48). An impaired cell mediated response and reduced levels of leptin have both been found to be features of low body weight in humans (49, 50).

Lord et. al. (46) found that leptin has a specific effect on T-cell responses, differentially regulating the proliferation of naïve and memory T-cells. They also tested the possibility of leptin influencing T_H cytokine production by measuring IL-2, INF- γ , and IL-4 levels in MLR experiments (46). They found that leptin increased IL-2 production and showed a substantial increase in INF- γ secretion. IL-4 production was completely inhibited, suggesting that leptin may bias T-cell responses towards a T_H1 phenotype and suppress the T_H2 phenotype. In the absence of leptin, T_H2 responses were dominate, the addition of leptin seemed to induce a switch from a T_H2 response to a T_H1 response. They also found that T-cells, rather than accessory cells, were the target of leptin action. Cognate recognition by T-cell antigen receptor was required before leptin could take effect. This indicated that the enhancement of proliferation observed was due to a specific effect of leptin receptor signaling rather than to a non-specific mitogenic stimulus (46).

Chapter 4: Materials and Methods

Mice

BALB/c mice were originally obtained from Harlan-Sprague Dawley, Inc. (Indianapolis, IN) and were bred in our own mouse colony (Department of Biology) . Both male and female mice were used with ages ranging from 6 to 12 weeks. The mice were housed in a light and temperature controlled environment with a light/dark cycles of approximately 12 hours each.

Leishmania Infection and Maintenance

The *Leishmania major* (Freidlin strain, NIH) pathogen was originally provided by Dr. D. Sacks. The pathogenicity of the *Leishmania* was maintained by passage through BALB/c mice on a regular basis. To accomplish this, the mouse was infected via the left rear footpad with about 1.0×10^6 organisms per ml. The infection was allowed to progress for two months. These mice were used as the source of pathogenic *L. major* by removing the spleen and/or popliteal lymph node (located between the knee joint and the articular end of the femur) and culturing the cells. The intracellular amastigote form of *L. major* was released from the hosts' cells. These pathogens then developed into the infective promastigote form when grown *in vitro*. The promastigotes were grown at 25 °C and passed in culture approximately once every week as described below.

The media used to grow the pathogen was Dulbecco's M199 (Sigma Chemical Co., St. Louis MO) amended with 1M HEPES, 10 units penicillin, 10 µg/L streptomycin, 0.35g/L sodium bicarbonate, and 20% fetal calf serum. The pH of the medium was adjusted to 7.2. For all of the experiments, promastigotes, which had not been passed *in vitro* more than five times, were injected into the left hind footpad with a dose of 1.0×10^6 organisms per ml. Over a series of 7 weeks, the middle of the infected footpads were measured with a vernier caliper gauge. The regional draining lymph nodes (popliteal) were also palpated to indicate the spread of infection. Swelling of the popliteal lymph node was indicative of the spread of infection.

Cyclosporin A

Cyclosporin A (CsA) was originally obtained by Sandoz, Ltd., East Hanover, NJ. It arrived prepared as an oral solution at concentration of 100mg/ml in olive oil in a labrafil base. A group of 15 mice, about 12 weeks in ages were each given a dose of CsA at 75 µg/g of body weight twice daily for fourteen days, beginning one day prior to infection with *L. major* for both trials. The CsA was administered using a 20 µl pipetmen and a plastic tip. The CsA was squirted into the mouth of the mouse. The mice were weighed before the administration of the drug to determine the dose and to observe the effect of the drug on the weight of the mice. The health of the mice were observed by noting the general appearance of the animals, texture of the fur, and the evidence of diarrhea in the cages. The animals were observed for smooth, ruffled or oily fur.

Leptin

Murine leptin was purchased from Sigma Chemical Co., St. Louis, MO. The leptin used was in a solution of 0.2mg/ml in sterile PBS. During Trial 1, a group of 12 mice at 8 weeks in age were treated with 1.8 µg/g of body weight twice daily for five days, beginning two days prior to infection. During trial 2, a group of 15 mice at 8 weeks in age were treated with 2.0µg/g of body weight twice daily for five days, beginning two days prior to infection. The different rates were used due to the fact that level of leptin in the body determines the type of T_H response (39). The leptin was administered with a ½ cc insulin syringe with a 30 gauge needle. The injections were given intraperitoneally. The mice were weighed before the administration of the drug to determine the dose and to observe the effect of the drug on the weight of the mice. The health of the mice was observed by noting the general appearance of the animals, texture of the fur, and the evidence of diarrhea in the cages, as described above.

FK506

Fujisawa Pharmaceuticals USA, Inc., Aurora, IL generously donated the powdered form of FK506. The powder was reconstituted in 0.2% w/v carboxymethylcellulose (CMC) at a concentration of 2.5 mg/ml. The CMC was prepared in a 1% stock solution by dissolving CMC in sterile water. This solution was then diluted to creating a working solution at 0.2% w/v. A group of seven mice at an age of 8 weeks were used for the first trial and a group of 15 mice at an age of 8 weeks were used for the second trial. These mice were given a dose of 50 µg/g of body weight once daily for 7

days, beginning one day prior to infection. The FK506 was administered intraperitoneally using a 1 cc syringe and a 26-gauge needle. The mice were weighed before the administration of the drug to determine the dose and to observe the effect of the drug on the weight of the mice. The health of the mice were observed as previously described.

Therapeutic FK506

FK506 was also used in a small group of mice (5 mice) in a therapeutic treatment regimen. These mice, along with a control group (infected, non-treated), were infected with 1×10^6 *Leishmania* promastigotes/ml. Fourteen days post infection treatment with FK506 was given at a dose of 50 $\mu\text{g/g}$ of body weight intraperitoneally using a 1 cc syringe and a 26-gauge needle. Control mice were given 0.2% w/v CMC intraperitoneally with a 1 cc syringe and a 26-gauge needle. Both groups were treated once daily for seven days. Health was monitored as described previously.

Control Mice

A group of 20 mice at 16 weeks in age were used as a control group. These mice were given sterile 0.2% w/v CMC intraperitoneally for seven days beginning one day prior to infection. This dose was based on the amount of fluid that would have been given if they were in the FK506 group. It was administered via a 1cc syringe and a 26 gauge needle. The mice were weighed before the administration of the solution to

determine the dose and to observe the effect of the solution on the weight of the mice.

The health of the mice was observed as described previously.

Limiting Dilution Assay

The limiting dilution assay is based on techniques described by Buffet et al (51). One animal from each group was sacrificed either by cervical dislocation or by CO₂ asphyxiation. The spleen and popliteal lymph node was removed aseptically and placed in a sterile petri dish containing 2 ml of complete Dulbecco's M 199 medium. Twenty milligrams of each organ was aseptically weighed for use in the assay. To release the *L. major*, each organ was ground between two sterile frosted glass slides. The ground tissue was then placed into 20 ml of complete Dulbecco's M 199 medium to make a 1mg/ml suspension. A duplicate series of 12 sterile tubes containing 2.5 ml of complete Dulbecco's M 199 medium amended with 20% fetal calf serum was prepared. Zero point five milliliters of the stock suspension was placed in the first tube and then 1:6 serially diluted. The tubes were incubated at room temperature (23°C - 24°C) for at least 3 weeks. The presence of any *L. major* promastigotes microscopically at 400x indicated a positive result for that dilution and represented the minimal most probably number (MPN).

Flow Cytometry Analysis

The procedure used in the flow cytometry analysis of lymphocytes extracted from the popliteal lymph node was based on Barbara Foster and Calman Prussin's protocols

(52). Popliteal lymph nodes were removed from three animals in each being tested. The organs were ground between sterile frosted slides and washed in RPMI medium (52). Cells were suspended in complete, pre-warmed RPMI at a concentration of 2×10^6 cells/ml and split into polystyrene round bottomed tubes containing 2 ml each. Anti-CD3, anti-CD28 and *L. major* antigen was added to each tube, and the tubes were centrifuged at low speed (300xg) for five minutes to pellet the cells in close proximity with each other, the antibodies, and the antigen stimulant. Tubes were then incubated in the CO₂ incubator at 37 °C for 2.5 hours. A 200µg solution of BFA was added to each tube to prevent the export of intracellular cytokines being produced. Cells were then incubated for an additional 4 hours. After incubation, cells were washed with 0.1 M EDTA and transferred to 4 ml V-bottomed tubes. The cells were fixed in pre-warmed 4% Para-formaldehyde, washed again with cold PBS and then resuspended in 10% DMSO/PBS. Cells were counted and placed in freezer vials to be stored at -80 °C until staining could be performed.

The staining of stimulated, frozen cells was also adapted from Prussin's protocols (52). The cells were thawed in a 37 °C water bath for 10 minutes. Pre-warmed 25µl PBS-Saponin (PBS-S) was added to each sample of cells, and the cells were transferred to V-bottomed tubes. Cells were centrifuged for 10 minutes at high speed (1500xg), and the pellet was resuspended in PBS-S/milk. The milk is used to block non-specific binding proteins (52). The cells were then placed on ice for 30 minutes to allow the milk to act as

a blocking agent. One tube from each group was chosen as a control tube, and the contents of this tube split in-half. One-half of the cells were resuspended in unlabeled monoclonal antibodies against IL-4 and INF- γ . The other half was resuspended in non-specific rat IgG as an isotype control. The rest of the tubes were resuspended in PBS-S/milk. All tubes were placed on ice for 1 hour. After chilling, labeled antibodies against IL-4 and INF- γ were added to each tube, and the tubes were again placed on ice for 1 hour, in the dark. Cells were then washed in PBS-S and resuspended in PBS/BSA. Cells were counted in flow cytometer within 24 hours.

Chapter 5: Results

Effects of drug toxicity on the health of the mice is shown in Table 1. The infected, non-treated mice experienced mild weight loss, mild roughness of the coat, zero deaths, and mild diarrhea. The mice treated with CsA, which is known for its toxicity, experienced mild to severe weight loss, severe roughness of the coat, zero deaths, and severe diarrhea. After the treatment period ended, the overall appearance of these animals returned to normal. Mice treated with FK506 experienced very mild weight loss, very mild roughness of the coat, very mild diarrhea, and zero deaths. Leptin treated mice had symptoms that fell in-between the severity of the CsA treated mice and the mildness of the FK506 treated mice.

The measure of footpad thickness was used as an indicator used in determining disease process. Normal, healthy mice have an average footpad thickness of about 2mm. Figure 5 shows the average footpad thickness of mice in each treatment group and the non-treated infected group during Trial 1. The control group (non-treated) was challenged with *L. major* but not treated with any immunomodulatory drug. The mice in this group experienced a large amount of swelling that dropped off by week 17. This drop in footpad thickness was due to the large amount of ulceration. Which caused a loss in foot tissue.

During trial 2, the dose of leptin was increased from 1.8 $\mu\text{g/g}$ of body weight to 2.0 $\mu\text{g/g}$ of body weight. In the first trial, leptin seemed to delay the disease, however, in

Table 1 Drug Toxicity			
Drug Used	Observed Weight Loss	Smoothness of Coats by Visual Inspection	Overall Health by Visual Inspection
Controls (0.2% CMC)	Mild	Mild Roughness	Fair, 0 Deaths, Mild Diarrhea
CsA	Mild-Severe	Severe Roughness	Fair, 0 Deaths, Severe Diarrhea
FK506	Very Mild	Very Mild	Fair, 0 Deaths, Mild Diarrhea
Leptin	Mild	Mild Roughness	Fair, 0 Deaths, Mild Diarrhea

Table 1: Drug Toxicity. CsA was the most toxic of the drugs used, mice treated with this drug experienced severe diarrhea and mild to severe weight loss during the treatment period. Their fur was also severely ruffled. Mice treated with FK506 developed very mild diarrhea and very mild roughness of the fur. Leptin treated mice fell somewhere in-between, with mild weight loss, mild diarrhea, and mild roughness during the treatment period.

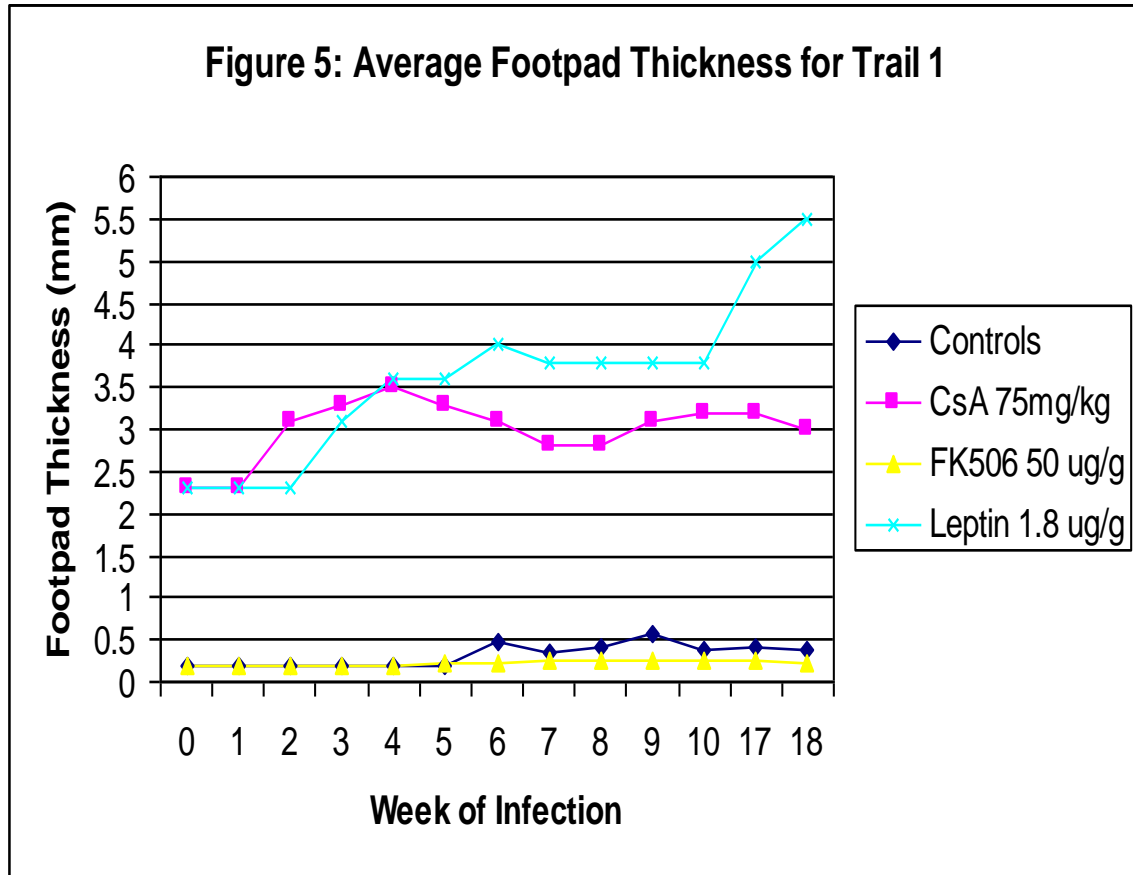


Figure 5: Average Footpad Thickness of Mice During Trial 1. Mice treated with FK506 developed very little swelling and no ulcers. Mice treated with CsA were similar to mice treated with FK506, little swelling occurred and no ulcer development occurred. Mice treated with leptin did experience more swelling and ulcer development. Non treated mice developed large amounts of swelling and ulcer development. The size of the footpads of the non-treated mice dropped due to necrosis of foot tissue.

the second trial the mice treated with leptin developed ulcers and swelling weeks before the non-treated animals. The mice treated with CsA experienced little swelling and no ulcer development. Mice treated with FK506 did not develop any swelling, redness, or ulcer development (Figure 6).

To determine parasite load, MPN dilution assays were performed on the draining regional lymph node and the spleen of animals from each group. Figure 7 presents average titers obtained from the popliteal lymph nodes. Titers were averaged together, data without standard error bars are an average of two animals, data with standard error bars are an average of four animals. Animals treated with FK506 did not have very high parasite loads. The parasite titer did drop in the FK506 animals by week 12. Animals treated with leptin had particularly high parasite titers, which also dropped by week 12. The parasite load in animals treated with CsA fluctuated throughout the experiment, but never reached levels as high as leptin animals or as low as FK506 animals. Infected, non-treated animals had parasite titers that increased fairly steadily with time. Only the spleens of animals in the infected non-treated group and the CsA group had positive titers. These positive results only occurred during weeks 6 and 12 (data not shown).

To determine the type of immune response occurring in the FK506 and in the infected non-treated animals, flow cytometry analysis of stained intracellular cytokines was used. Later, we also analyzed cells stained for intracellular cytokine production from all of the experimental groups. Figure 8 shows the percent of cells in each sample that

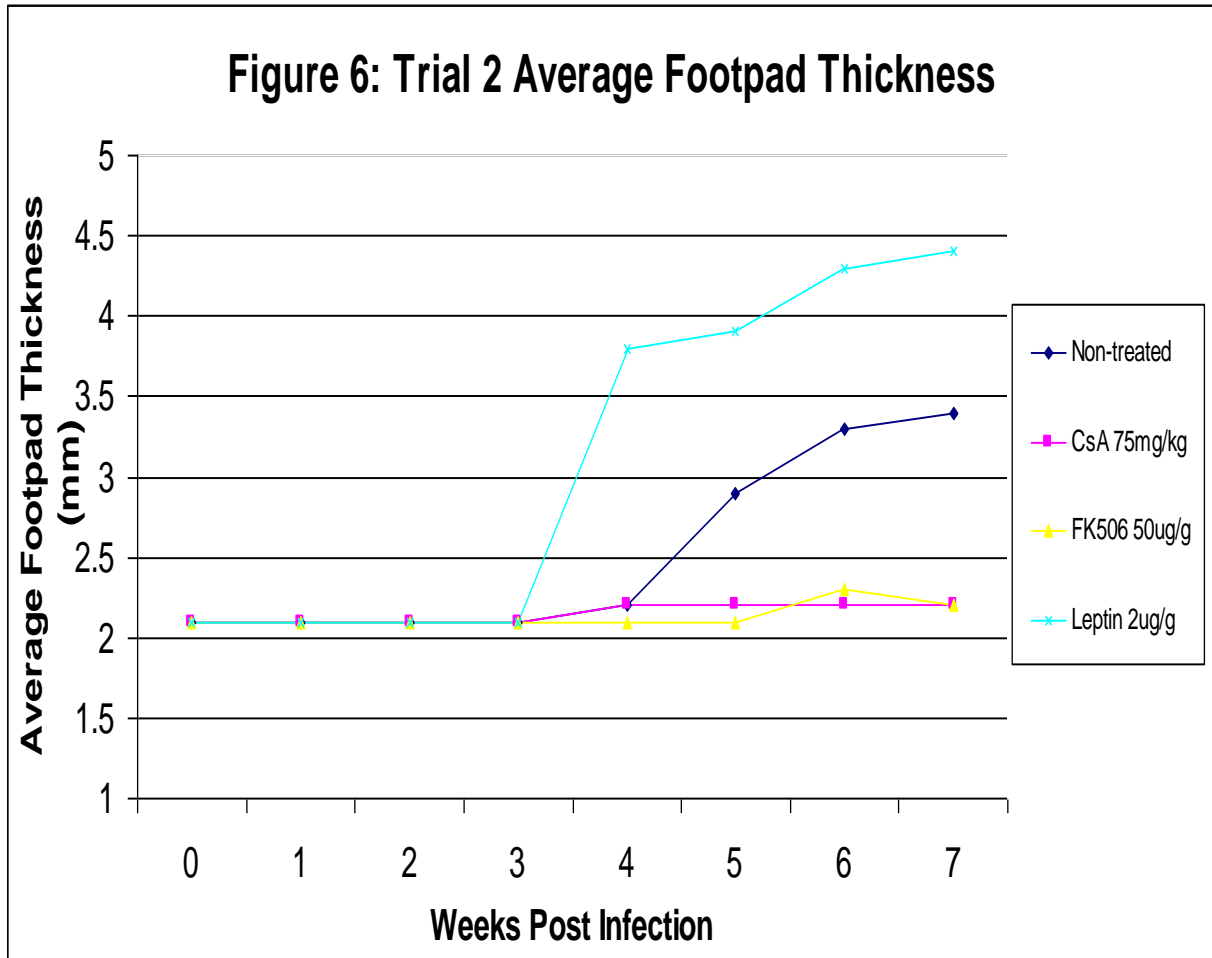


Figure 6: Average Footpad Thickness of Mice during Trial 2. FK506 and CsA treated mice did not have much swelling and did not develop ulcers. The mice in the leptin treated group experienced a large amount of swelling and ulcer development. The ulcer development occurred before the mice in the control, non-treated group.

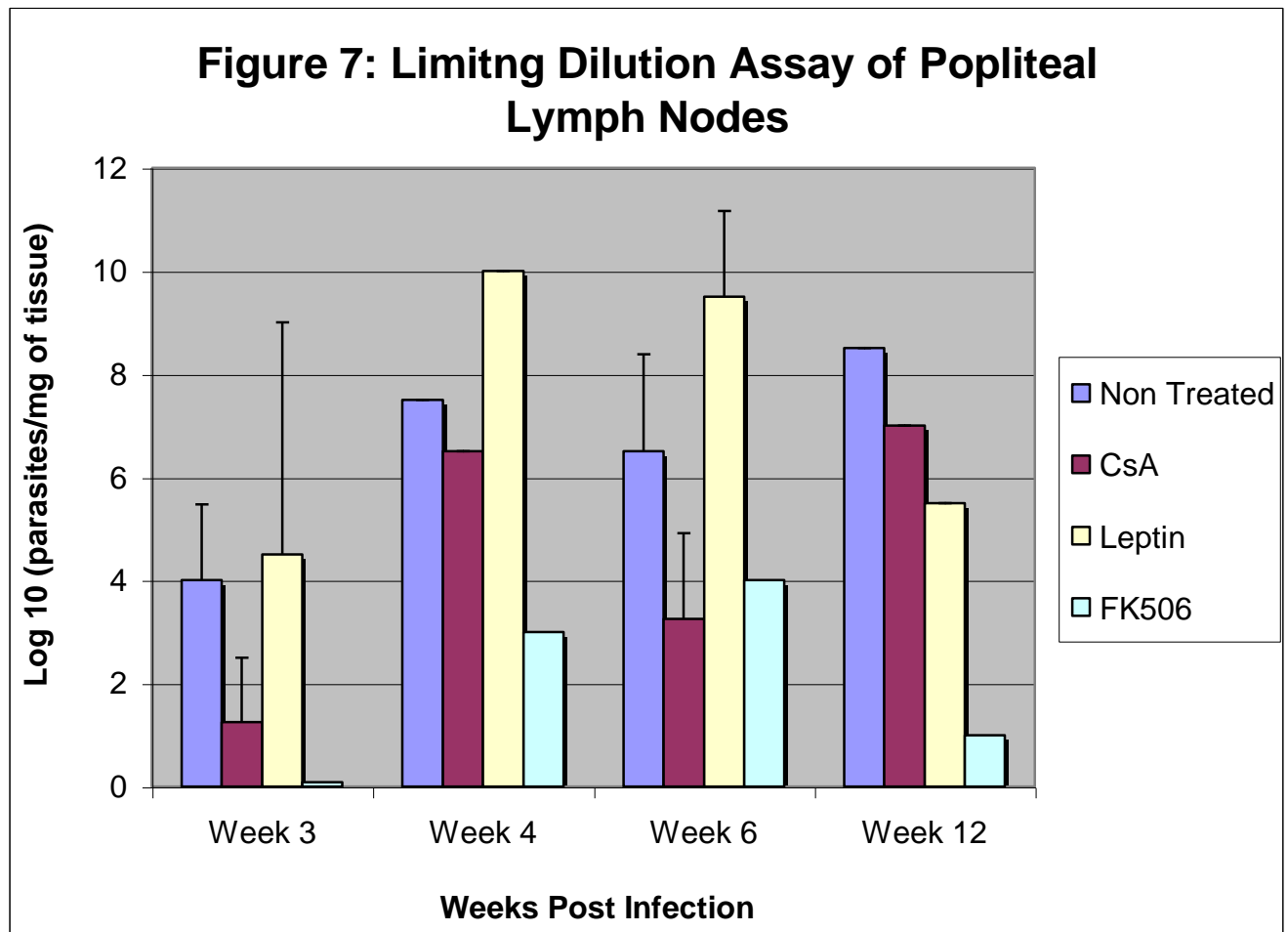


Figure 7: Limiting Dilution Assays of Popliteal Lymph Nodes. Data without error bars are an average of 2 animals, data with error bars are an average of 4 animals.

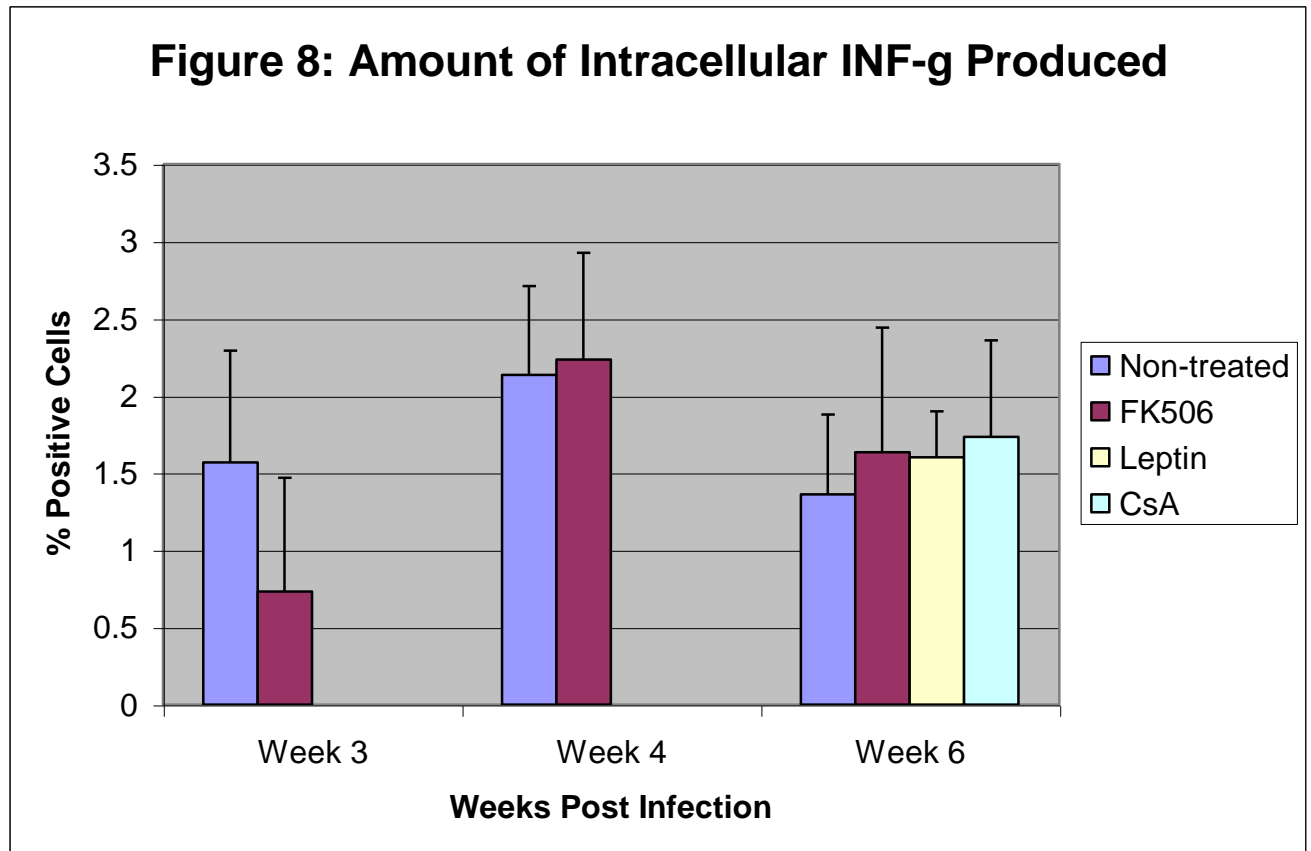


Figure 8: Amount of INF- γ produced by mice infected with *L. major*. Three weeks post infection, mice treated with FK506 were producing less INF- γ than mice that were infected but not treated. By four weeks post infection, the mice treated with FK506 were producing slightly more INF- γ . By six weeks post infection both the non-treated mice and the mice treated with FK506 were producing less INF- γ than the previous sample, however the treated mice were still producing more INF- γ . Mice treated with leptin were producing about the same amount of INF- γ as mice treated with FK506. Mice treated with CsA were producing the most INF- γ at six weeks post infection.

were positive for the production of INF- γ ; Figure 9 shows the percent of cells in the sample that were positive for IL-4.

Three weeks post infection; animals treated with FK506 were producing less INF- γ and IL-4 than infected non-treated animals. These animals were immunosuppressed. By four weeks post infection, animals treated with FK506 were producing more INF- γ and less IL-4 than infected non-treated animals, shifting the immune response toward a T_H1 type response. Six weeks post infection non-treated animals were producing the least amount of INF- γ and IL-4. Mice treated with leptin were producing the most amount of IL-4, indicating a strong T_H2 type immune response. Mice treated with FK506 or CsA were producing similar amounts of INF- γ , however the mice treated with FK506 were producing more IL-4 than mice treated with CsA. The mice treated with FK506 never appeared to be ill; their feet did not swell or develop ulcers. The only sign of illness was a swollen popliteal lymph node and a low titer of parasite from that same lymph node.

FK506 was not found to be effective as a therapeutic drug with treatment until 27 days post infection (Figure 10). The footpads of the treated mice and the non-treated mice began swelling at about the same time. The non-treated mice did experience slightly more swelling than the treated mice.

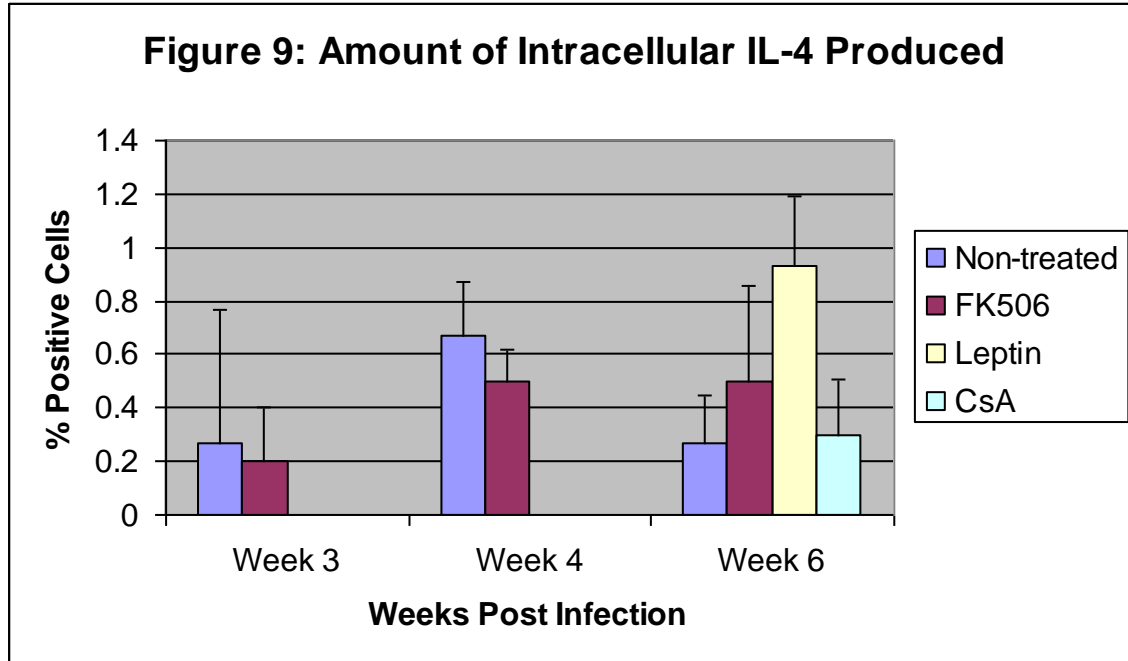


Figure 9: Amount of IL-4 Produced. Non-treated infected mice produced more IL-4 than mice treated with FK506 in weeks three and four post infection. Mice treated with leptin produced a large amount of IL-4 at six weeks post infection. These mice were very ill, with large foot pads, large ulcers, and large parasite loads.

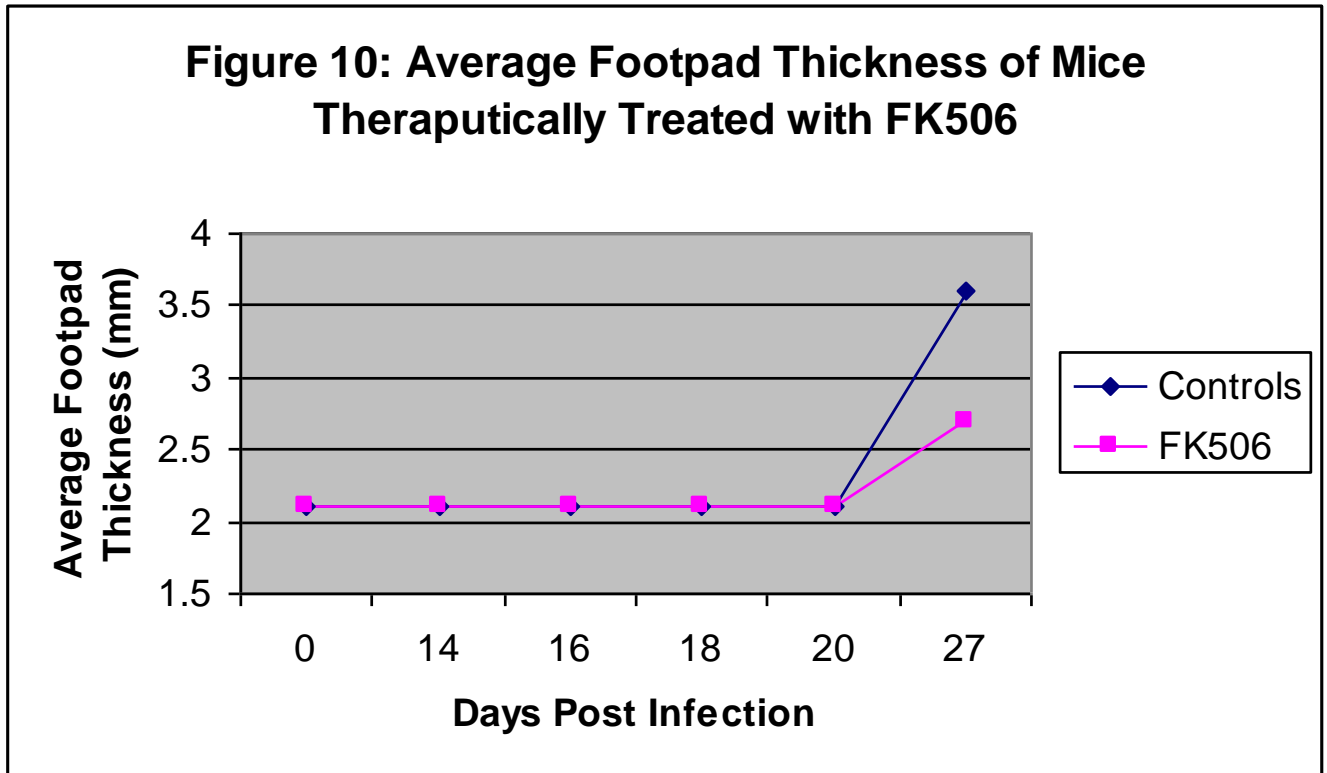


Figure 10: Average Footpad Thickness of Mice Therapeutically treated with FK506.

Treated and non-treated mice both developed footpad swelling at about the same time.

The treated mice displayed a smaller amount of swelling, but not by a large amount.

Chapter 6: Discussion

FK506 appears to be a very effective drug in preventing severe illness in BALB/c mice due to *Leishmania major* infection. During both trials, mice treated with FK506 did not show any signs of illness. The footpads of these mice only swelled a small amount. No ulcers were observed during either trial. As shown in Figure 5, the amount of parasites present in these mice was also much less than mice undergoing other treatment regimens. These mice were shown to be immunosuppressed three weeks after infection, producing very little of either cytokine (Figure 8 and Figure 9). Four weeks post infection these mice were producing much more INF- γ than the non-treated mice and much less IL-4 than the non-treated mice (Figure 9). By six weeks post infection the mice treated with FK506 had slowed down production of INF- γ while IL-4 production remained stable, however, the amount of INF- γ was still greater than the amount of IL-4 being produced (Figure 8 and Figure 9). This suggests that the suppression of the immune response early in the disease process inhibited illness. It also suggests that the type of immune response that was occurring later in the disease process was a T_H1 type response.

Due to the success of the first trial, a therapeutic experiment was performed for FK506. Results indicated that this particular treatment plan did not prevent disease (Figure 10). Both sets of mice experience footpad swelling at about day 27 post

infection. At this time, neither set of mice had developed ulcers. Based on the evidence provided in the prophylactic intracellular cytokine data, I believe that if the therapeutic treatment had been started earlier this result may have been very different. I also believe that the response would have been greater had we allowed the experiment to progress longer.

The cyclosporin A group of mice suggested that CsA is effective in preventing the mice from developing a severe reaction against the *Leishmania* infection. These mice experienced weight loss during the treatment period, but readily gained weight after treatment ended. CsA is a toxic drug that has caused death in previous treatment regimens (unpublished data), however with the treatment regimen used in both trials, no deaths occurred. Treated mice displayed some ruffling of fur during the treatment period, indicating illness caused by toxicity (Table 1). In the second trial we again saw similar toxicity data compared to the first trial. Although lower doses of cyclosporine may have reduced toxicity, unpublished data suggests that they would be below therapeutically beneficial levels.

These mice experienced some swelling of the footpad, which decreased with time (Figure 5 and Figure 6). They also experienced high titers of parasites in their lymph nodes, which also seemed to decrease as time passed (Figure 7). We observed cytokine production only in week six, and again did not see a major difference between the CsA treated animals and the infected, non-treated animals. Mice treated with CsA produced

low levels of both cytokines; however, compared to the non-treated animals they were producing more INF- γ (Figure 8). These mice produced about the same amount of IL-4 as the infected non-treated mice (Figure 9). The larger INF- γ production suggests that treatment with CsA pushes the immune response toward a T_H1 type response.

Mice in the leptin group fell somewhere between resistance and delay of illness during the first trial. They did not experience any weight loss or ruffling of the fur (Table 1). However they did appear to have an increased amount of footpad swelling and ulcer development (Figure 5). They also exhibited a large titer of parasites in their lymph nodes but small titer in their spleens. In the second trial the dose of leptin was increased. During the second trial the balance between T_H1 and T_H2 seemed to be shifted. Mice developed ulcers and a large amount of footpad swelling before the infected non-treated animals (Figure 6). Again, we observed cytokine production only in week six, and did not see a significant difference between the leptin treated animals and the infected non-treated animals. Animals were producing more IL-4 than INF- γ six weeks post infection (Figure 8 and Figure 9). The production of more IL-4 would indicate that leptin was pushing the immune response towards a T_H2 type response.

Based on the data presented, I believe that the immune response against *Leishmania major* infection in BALB/c mice is responsible for either systemic disease or resistance to the disease. Mice given FK506, which suppressed the immune system early in the infection, never showed signs or symptoms of illness (Table 1, Figures 5, 6, 7, 8,

and 9). The footpads of these mice did not swell or develop ulcers. Their fur remained smooth, and their parasite load remained low. Data suggests that resistance to leishmaniasis is determined early after infection. Future experiments should assay cells from each group of animals earlier in the disease process. I would also recommend that therapeutic treatment using FK506 begin earlier after infection.

Chapter 7: References

1. Foote, Simon J., Hardman, Emanuel, and Roberts, Lynden J.(2000). Leishmaniasis. *BMJ*, 321:801-804.
2. Behforouz, Nancy C., Mathison, Barry A., and Wenger, Charlotte D. Mathison, Barry A. (1986). Prophylactic Treatment of BALB/c Mice with Cyclosporine A and Its Analog B-5-49 Enhances Resistance to *Leishmania major*. *J. of Immunol.* 136:3067-3075.
3. Roberts, Larry S., and Schmidt, Gerald D. (2000). *Foundations of Parasitology*. Fourth Edition
4. Conceicao-Silva, Fatima, Hahne, Michael, Louis, Jacques, Schroter, Michael, L., and Tschopp, Jurg. (1998) The Resolution of Lesions Induced by *Leishmania major* in Mice Requires a Functional FAS (APO-1, CD95) Pathway of Cytotoxicity. *Eur. J. Immunol.* 28: 237-245.
5. Bang, H., Bang, R., Brune, K., Hoerauf, A., Pahl, A., Rascher, C., Rollinghoff, M., and Solbach, W. (1997) Host-cell Cyclophilin is Important for the Intracellular Replication of *Leishmania major*. *Mol. Microbiol.* 24: 421-429.
6. Benjamini, Eli, Leskowitz, Sidney, and Sunshine, Geoffrey (1996). *Immunology a Short Course*. Third Edition.
7. Kindt, Thomas J., Goldsby, Richard A., and Osborne, Barbara A. (2000). *Kuby Immunology*. Fourth Edition.
8. Fernandez-Botran, R., Mosmann, T. R., Sanders, V. M., and Vitetta, E. S. (1988). Lymphokine-mediated Regulation of the Proliferative Response of Clones of T helper 1 and T helper 2 Cells. *J. Exp. Med.* 168: 543-558.
9. Davis, Kenneth A., Larenas, Patricia V., Sornass, Thierry, deVries, Jan E., and Yssel, Hans (1996). Differentiation and Stability of T Helper 1 and 2 Cells Derived from Naïve Human Neonatal CD4⁺ T Cells Analyzed at the Single Cell Level. *J. Exp. Med.* 184: 473-483.
10. Georgion, Andrew, Hussell, Tracy, O'Garra, Anne, Openshaw, Peter J.M., and Spender, Lindsay C. (1996). T_H1 and T_H2 Cytokine Induction in Pulmonary T Cells During Infection with Respiratory Syncytial Virus. *J. General Virol.* 77: 2447-2455.
11. Field, E. H., Goeken, J., Noelle, R. J., Rouse, T., and Waldschmidt, T. (1993) Evidence for Excessive Th2 CD4⁺ Subset Activity in vivo. *J. Immunol.* 151: 48-59.
12. Bentwich, Zvi, Kalinkovich, Alexander, and Weisman, Ziva. (1995). Immune Activation is a Dominant Factor in the Pathogenesis of African AIDS. *Immunol. Today* 16:187-191.

13. Boom, W. H., Hazlett, F. E. Jr, Kazura, J. W., and Pearlman, E. (1993). Modulation of Murine Cytokine Responses to Mycobacterial Antigens by Helminth-Induced T helper 2 Cell Responses. *J. Immunol.* 151: 4857-4864.
14. Maizels, Rick M., and Yazdanbakhsh, Maria. (2003). Immune Regulation by Helminth Parasites: Cellular and Molecular Mechanisms. *Nature Reviews Immunology* 3: 733-744.
15. Bogdan, C., Rollinghoff, M., Stenger, S., Thuring, H. (1994). Tissue Expression of Inducible Nitric Oxide Synthase is Closely Associated with Resistance to *Leishmania major*. *J. Exp. Med.* 180: 783-793.
16. Galchenhaus, Nicolas, Julia, Valeria, and Rassoulzadegan, Minoo (1996). Resistance to *Leishmania major* Induced by Tolerance to a Single Antigen. *Science* 274:421-423.
17. Locksley, R. M., Mallet-Designé, V., Mohrs, M., Stetson, D. B., and Teyton, L. (2002). Rapid Expansion and IL-4 Expression by *Leishmania*-specific Naïve Helper T cells in vivo. *Immunity* 17: 191-200.
18. Himmerlich, H., Launois, P., Louis, J. A., and Tacchini-Cottier, F. (1999). Some of the Early Events Underlying Th2 Cell Maturation and Susceptibility to *Leishmania major* Infection in BALB/c Mice. *Biol. Chem.* 380: 909-914.
19. Glaichenhaus, Nicolas, and Schilling, Sabrina (2001). T Cells that React to the Immunodominant *Leishmania major* LACK Antigen Prevent Early Dissemination of the Parasite in Susceptible BALB/c Mice. *Infect. and Immunity.* 69: 1212-1214.
20. Greenwald, Rebecca J., McAdam, Alexander J., Satoskar, Abhay R., and Sharpe, Arlene H., and Wander Woude, Diane (2002). Cutting Edge: Inducible Costimulatory Protein Regulates Both T_H1 and T_H2 Responses to Cutaneous Leishmaniasis. *J. of Immunol.* 2:991-995.
21. Traber, Rene` and VonWartburg, Albert (1988). Cyclosporins, Fungal Metabolites with Immunosuppressive Activities. *Progress in Medicinal Chemistry.* 25:1-33.
22. Kleinkauf, H., and Von Dohren H. (1996). A Non-Ribosomal System of Peptide Biosynthesis. *Eur. J. Biochem.* 236: 335-351.
23. Kincaid, Randall, L., O'Keefe, Stephen, J., O'Neill, Edward A., Tamura, Jun'ichi, and Tocci, Michael J. (1992). FK506 and CsA-Sensitive Activation of the IL-2 Promoter by Calcineurin. *Nature* 357:692-694.
24. Liu, J. et. al. (1991) Calcineurin is a Common Target of Cyclophilin-Cyclosporin A and FKBP-FK506 Complexes. *Cell* 66:807-815.
25. Aoki, Hatsuo, Goto, Toshio, Hatanaka, Hiroshi, Hashimoto, Michisane, Kino, Toru, Kohsaka, Masanobu, Imanaka, Masanobu, Nishiyama, Michihisa, and Okuhara, Masakuri (1987). FK506: A Novel Immunosuppressant Isolated from a *Streptomyces*. I. Fermentation, Isolation, and Physio-Chemical and Biological Characteristics. *J. of Antibiotics.* 9:1249-1255.

26. Aoki, Hatsuro, Goto, Toshio, Hatanaka, Hiroshi, Inamura, Noviaki, Kino, Toru, Kohsaka, Masanobu, Miyata, Susumu, Nishiyama, Michihisa, Ochiai, Takenori, Okuhara, Masakuni, and Yajima, Toshimi (1987). FK506: A Novel Immunosuppressant Isolated from a *Streptomyces* II. Immunosuppressive Effect of FK506 in Vitro. *J. of Antibiotics*. 9: 1256-1264.
27. Blazar, Bruce R., Fitzsimmons, William E., Taylor, Patricia A., and Vallera, Daniel A. (1994). FK506 Inhibits Graft-Versus-Host Disease and Bone Marrow Graft Rejection in Murine Recipients of MHC Disparate Donor Grafts by Interfering with Mature Peripheral T Cell Expansion Post Transplantation. *J. of Immunol*. 94: 1836-1846.
28. Aldskogius, H., and Tao, R. (1998). Influence of FK506, Cyclosporin A, Testosterone, and Nimodipine on Motoneuron Survival Following Axotomy. *Restorative Neurology and Neuroscience*. 12: 239-246.
29. Cyert, Martha S., Botstein, David, Brown, Patrick O., Gasch, Audrey P., Li Hong, Xia, Ogawa, Nobuo, Saltsman, Kirstie, and Yoshimoto, Hiroyuki (2002). Genome-wide Analysis of Gene Expression Regulated by the Calcineurin/Crz1p Signaling Pathway in *Saccharomyces cerevisiae*. *J. Biol. Chem*. 277: 31079-31088.
30. Hunter, Daniel, Lowe, James B., Mackinnon, Susan E., Sen, Subhro K., Sobol, Julia B., and Yang, Robin K. (2003). Effects of Delaying FK506 Administration of Neurogeneration in a Rodent Model. *J. of Reconstructive Microsurgery*. 19: 113-118.
31. Dawson, T.M., Lyons, W. E., and Steiner, J.P., et. al. (1994). The Immunophilins, FK506 Binding Protein and Cyclophilin are Discretely Localized in the Brain: Relationship to Calcineurin. *Neuroscience*. 62: 569-580.
32. Dawson, T. M., Lyons, W.E., Snyder, S. H., and Steiner, J.P. (1995). Neuronal Regeneration Enhances the Expression of the Immunophilin FKBP-12. *J. of Neuroscience*. 15:2985-2994.
33. Agostinho, Paula and Resende-Oliveira, Catrina. (2003). Involvement of Calcineurin in the Neurotoxic Effects Induced by Amyloid-beta and Prion Peptides. *Euro. J. of Neuroscience*. 17:1189-1196.
34. Prusiner, S.B. (1996). Molecular Biology and Pathogenesis of Prion Diseases. *Trends Biochem. Sci*. 252: 482-487.
35. Fei, H., Lee, G. H., Maffei, M., et. al. (1995). Increased Expression in Adipocytes of obRNA in Mice with Lesions of the Hypothalamus and with Mutations at the db Locus. *Proc. Natl. Acad. Sci. USA* 92: 6957-6960.
36. Boguski, M.S. and Bryant, S.H., and Madej, T. (1995) *FEBS Cell* 373:13
37. Goberna, Raimundo, Santos-Alvarez, Jose', and Sanchez-Margalet, Victor. (1999). Human Leptin Stimulates Proliferation and Activation of Human Circulating Monocytes. *Cellular Immunol*. 194:6-11.
38. Goberna, Raimundo, Martin-Romero, Consuelo, Sanchez-Margalet, Victor, and Santos-Alvarez, Jose` (2000). Human Leptin Enhances Activation and

- Proliferation of Human Circulating T Lymphocytes. *Cellular Immunol.* 199:15-24
39. Fontana, Silvia, La Cava, Antonio, Lechler, Robert I., Lord, Graham, Matarese, Guiseppe, Sanna, Veronica, and Zappacosta, Serfino (2002). Balancing Susceptibility to Infection and Autoimmunity: A Role for Leptin? *Trends in Immunol.* 23:182-187.
 40. Matarese, G. et. al. (2001). Requirement for Leptin in the Induction and Progression of Autoimmune Encephalomyelitis. *J. of Immunol.* 166:5909-5916.
 41. Chandra, R.K. (1996). Nutrition, Immunity and Infection: From Basic Knowledge of Dietary Manipulation of Immune Responses to Practical Application of Ameliorating Suffering and Improving Survival. *Proct. Natl. Acad. Sci. USA.* 93:14304-14307.
 42. Whitacre, G.J. (2001). Sex Differences in Autoimmune Disease. *Nat. Immunol.* 2: 777-780.
 43. Friedman, J. M., and Halaas, J. L. (1998). Leptin and the Regulation of Body Weight in Mammals. *Nature* 395: 763-770.
 44. Campfields, L. A., Clark, F. T., Culpepper, J., Deeds, J., Dembski, M., Deng, N., Devos, R., Mays, G. G., Monroe, C. A., Moore, K.J., Moriarty, A., Muir, C., Richards, G. J., Sanker, S., Smutko, J. S., Tartaglia, C. A., Tepper, R. I., and Woolf, E. A. (1995). Identification and Expression Cloning of a Leptin Receptor, OB-R Cell 83:1263-1271
 45. Bailon, P. S., Baumann, H., Dembski, M., Kim, H., Lai, C. F., Morella, K. K., Tartaylia, L. A., and White, D. W. (1996). The full-length leptin receptor has signaling capabilities of interleukin 6-type cytokine receptors. *Cell Bio.* 93:8374-8378.
 46. Baker, Richard J., Bloom, Stephen R., Howard, Jane K., Lechler, Robert I., Lord, Graham M., and Matarese, Giuseppe (1998). Leptin Modulates the T-cell Immune Response and Reverses Starvation Induced Immunosuppression. *Nature* 194:897-901.
 47. Boden, G., Chen, X., Mozzoli, M., and Ryan, I. (1996) Effect of Fasting on Serum Leptin Levels in Normal Human Subjects. *J. Clin. Endocrinol. Metab.* 81: 3419-3423.
 48. Garraf, P., et. al. (1997). Multiple Cytokines and Acute Inflammation Raise Mouse Leptin Levels: Potential Role in Inflammatory Anoxeria. *J. Exp. Med.* 185: 171-175.
 49. Polack, E., et. al. (1993). Low Lymphocyte Interferon-gamma Production and Variable Proliferation Response in Anorexia Nervosa Patients. *J. Clin. Immunol.* 13: 445-451.
 50. Grinspoon, S., et. al. (1996). Serum Leptin Levels in Women with Anorexia Nervosa. *J. Clin. Endocrinol. Metab.* 81: 3861-3863.
 51. Buffet, P. A., Derouin, F., Garin, Y. J. F., Nassar, N., and Sulahian, A. (1995). Culture of Microtitration: A Sensitive Method for Quantifying *Leishmania*

- infantum* in Tissues of Infected Mice. Antimicrobial Agents and Chemotherapy. 39: 2167-2168.
52. Foster, Barbara and Prussin, Calman (2002) Current Protocols in Immunology 6.24.1-6.24.16